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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,222,993**, on February 4, 1998, by **THE ONTARIO CANCER INSTITUTE,** assignee of Jean Gariépy and Mark Robert Bray, for "A Method for Using a Ribosome-Inactivating Protein Complex as a Structural Template and a Molecular Search Engine in the Design, Construction and Screening of Combinatorial Protein Libraries".

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Date

A Method for Using a Ribosome-inactivating Protein Complex as a Structural Template and a Molecular Search Engine in the Design, Construction and Screening of Combinatorial Protein Libraries

FIELD OF THE INVENTION

The invention is a method for identifying new therapeutic or diagnostic proteins capable of binding to a target cell line, wherein the new proteins are derived by mutating a binding subunit of a wild type heteromeric protein to create a library of mutant proteins which are then screened for their ability to specifically bind to and kill a target cell line.

BACKGROUND OF THE INVENTION

Most present-day chemotherapeutic agents used in controlling eukaryotic cell proliferation (as exemplified by anticancer and antifungal agents) tend to be small molecules that are able to perform a single task relatively well, i. e., killing or arresting the proliferation of rapidly dividing cells. Unfortunately, most of these chemotherapeutics possess minimal tissue specificity and non-optimal biodistribution profiles. Shiga toxin and related toxins are members of a family of ribosome-inactivating proteins (RIPs) that are potent inhibitors of protein synthesis in eukaryotic cells. Shiga toxin is composed of six subunits: one enzymatic A subunit responsible for inactivating protein synthesis and a pentamer of identical B-subunits that encode for the toxin's specificity for surface receptors (CD77; Gb_3) present on eukaryotic cells.

The present invention utilizes the concept of using a multi-tasking protein toxin such as Shiga toxin (or other related RIP) as a molecular template in developing powerful cytotoxic agents having the ability to bind specifically to target cells. By modifying residues affecting only the receptor-binding specificity of the toxin template, it is possible in accordance with the invention to use the toxic A subunit present in all mutant toxins as a molecular search engine in screening combinatorial protein libraries of the toxin's template to find mutant toxins that kill specific cell lines or cell types. Using the method of the invention, it has been shown that a family of related mutant combinatorial toxins can be derived that can kill breast cancer cell lines which were previously insensitive to the native toxin.

Since the receptor binding potential of combinatorial proteins such as Shiga toxin (B-subunit pentamer) can be dissociated from its cytotoxic A subunit, the potential also exists for developing non-cytotoxic, diagnostic probes for detecting the presence of useful cell surface markers to aid in the selection of therapeutic strategies. The data presented herein support the broad potential of combinatorial Shiga toxin libraries (or libraries of any RIP member) as sources of potentially cell-specific cytotoxic and diagnostic agents.

SUMMARY OF THE INVENTION

The invention provides a method for identifying therapeutic or diagnostic proteins capable of binding specifically to a target cell line, which said proteins are derived from a wild type heteromeric protein having a cell surface binding subunit and a cytotoxic subunit, comprising the steps of: a) creating libraries of mutant

heteromeric proteins in which the cell binding subunit has been randomly mutated at known binding sites; and b) screening the library (using the cytotoxic domain present in all mutant toxins as a built-in search engine) with a target cell line which is insensitive to the wild type protein and identifying those mutants which kill the cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the amino acid sequences of the A and B subunits of Shiga-like toxin 1. Panel A shows the catalytic A subunit. Panel B shows the B subunit with the three boxed regions representing loops harbouring residues postulated to be involved in creating a receptor binding cleft for CD77.

Figure 2 shows backbone representations of Shiga toxin (ShT; panel A, side view) and its B subunit (panels B and C, bottom view). ShT and SLT-1 have identical B subunits. The catalytic A subunit (in magenta, Panel A) has its C-terminus inserted into the central hole of the B subunit pentamer (in green). The B subunit pentamer (in green, Panel B) is stabilized by intra- and inter-subunit interfaces involving ß-sheets. Two of the three loop regions of the B subunit boxed in Fig. 1 (residues 15-19 and 30-33) are highlighted in red to show the orientation and location of these loops in relation to the ß-strand structure of the B subunit and the A chain itself. Loop 58-66 is located in the same vicinity as loops 15-19 and 30-33 and was not highlighted for reasons of clarity. In Panel C, each identical B subunit is coloured differently to illustrate their symmetrical arrangement giving rise to a pentamer. Most of the A chain was coloured in grey to simplify the drawing.

Figure 3 shows oligonucleotide sequences of primers

synthesized for creation of the ShT libraries. Loop 1 and loop 2 represent residues 15-19 and 30-33 of the B subunit, respectively. Primer **A** was synthesized to have controlled levels of randomization in the two loops as described in the text. Primer **B** overlaps primer **A** by 15 bases at its 3´ end, and was used to create a combinatorial cassette by mutually primed synthesis in conjunction with primer **A**. Restriction sites used to clone the libraries are indicated in bold.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Many plant and bacterial toxins represent successful protein designs able to penetrate mammalian cells and localize themselves into intracellular compartments. These proteins are very effective at deleting target cells or at activating non-lethal cellular processes. The understanding of how such proteins are constructed to perform intracellular tasks has increased dramatically in the last two decades with the overall consequence that one may now view such protein designs as potential therapeutic constructs. A large number of these toxins can be grouped under a common theme of structural organization. They are heteromeric in nature with two or more polypeptide domains or subunits responsible for distinct functions (1). In such proteins, the two or more subunits or domains could be referred to as A and B, and the toxins as AB_x toxins where *x* represents the number of identical or homologous B subunits in the toxin. This family of framework-related toxins includes examples such as Shiga and Shiga-like toxins, the *E. coli* heat-labile enterotoxins, cholera toxin, diphtheria toxin, pertussis toxin, *Pseudomonas aeruginosa* exotoxin A (2,3) as well as plant toxins such as ricin and abrin.

Based on their ability to block protein synthesis, proteins such as Shiga and Shiga-like toxins as well as ricin, abrin, gelonin, crotin, pokeweed antiviral protein, saporin, momordin, modeccin, sarcin, diphtheria toxin and exotoxin A have been referred to as ribosome-inactivating proteins (RIP). The potency of RIPs is exceedingly high; one molecule of diphtheria toxin A chain (99) or ricin A chain (100) having been shown to be sufficient to kill a eukaryotic cell. The crystal structures for many of these molecules have now been established (4-12), and insights into their functions have mostly focused on the identification of residues involved in the catalytic activity of A chains and on mapping B subunit residues involved in receptor-binding activity. It has become clear that the quaternary structure of RIPs codes for multiple functions; their ability to bind to defined surface receptors, to be internalized and routed inside cells, to be activated by intracellular processing events, and to translocate their catalytic domain near their target substrate. However, the delimitation of which parts of these toxins perform which tasks is not completely known.

Shiga (ShT) and Shiga-like (SLT) toxins possess the smallest known B subunit (less than 70 residues) of all AB_x toxins, and their A subunit has an identical catalytic activity as the corresponding subunit in ricin.

Mode of action of Shiga and Shiga-like toxins

Shiga toxin (ShT) and Shiga-like toxins (SLT) are structurally related bacterial toxins involved in the pathogenesis of bacillary dysentery, hemorrhagic colitis, the hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (19-21). Shiga toxin, the first member of this family of cytotoxins to be reported in

1903 (22,23) is produced by *Shigella dysenteriae 1*. Shiga-like toxins have been recently identified as virulence factors elaborated by enterohemorrhagic strains of *E. coli* (24-28). In particular, the *E. coli* strain O157:H7, which produces Shiga-like toxin 1, has been recently identified as the causative agent responsible for recent mass outbreaks of food poisoning in Japan and the United States.

These toxins are proteins composed of six subunits; one catalytic A subunit (293 amino acids; MW 32,317) involved in the blockage of protein synthesis and five B subunits (69 amino acids; MW 7600 each) necessary for the attachment of the toxin to cells (29-35; Figure 2). The B subunits spontaneously assemble into a pentamer in solution (Figure 2, panels B and C). The structure of these toxins typifies a common motif employed by other larger bacterial toxins such as cholera toxin and the *E. coli* heat-labile enterotoxins (6,7) and pertussis toxin (8). However, the mode of action of members of the ShT family of toxins is quite different. The cell specificity of ShT and SLT-1 is encoded by its B subunit which recognizes the glycolipid globotriaosyl ceramide (referred to as CD77 or Gb_3; Galα1→4Galβ1→4Glcβ1→ 1Ceramide; ref. 36,37). Following its attachment to susceptible cells, Shiga toxin is endocytosed from coated pits (38-40). The A-chain is processed to a smaller 27 kDa A_1 fragment through a selective nicking and reduction of the native chain. The A_1 fragment is responsible for the inactivation of eukaryotic ribosomes (29) acting as a highly specific N-glycosidase which cleaves a single adenine residue from 28S rRNA (41,42). Depurination at that site inhibits peptide elongation by preventing the EF-1 dependent binding of aminoacyl tRNA to the 60S ribosomal subunit (43-45).

Construction and screening of Shiga toxin libraries to derive useful diagnostic and therapeutic agents targ ted at defined eukaryotic cell populations.

A principal objective of this invention is to develop therapeutically useful Shiga toxin variants that will bind to surface markers (glycolipids, glycoproteins, or proteins, as examples) expressed on human tumour cells in preference to normal cells. Alternatively, toxin variants could be found to target any defined eukaryotic cell populations such as pathogenic fungi or to control the growth of rapidly proliferating cells (implicated in scar management, tissue remodelling, or skin diseases for example). In accordance with the invention, the receptor specificity of the toxin, which is encoded by its B subunit, is altered by random mutagenesis. Mutations in the B subunit must be kept to a minimum in order to lessen any negative effects on other functions of the toxin such as the toxicity of its A chain and the proper folding and assembly of the holotoxin (i. e., pentamerization of the B subunit, insertion of the A_2 domain into the B pentamer, exposure and orientation of the protease sensitive loop, and packing environment of the translocation domain).

Shiga and Shiga-like toxin 1 have identical B subunits. The B subunit is a small protein composed of only 69 amino acids that pentamerizes spontaneously in solution. Its crystal structure (as a pentamer of B subunits) has been solved in the presence and absence of the A subunit (4,5) and has been shown to be identical in either context. Each B subunit monomer within the pentameric structure is composed of 6 ß-strands (ß1, residues 3-8; ß2, residues 9-14; ß3, residues 20-24; ß4, residues 27-31; ß5, residues 49-53; ß6, residues 65-68) involving 31 of its 69 amino acids (45% ; Fig. 2). A

single α-helix (residues 36 to 46) accounts for 16% of the remaining structure. These elements of secondary structure appear essential for the maintenance of the pentamer integrity and its association with the A_2 domain of the A chain (Fig. 2). Thus, any perturbations in these regions may result in folding problems. Three loop regions composed of more than two amino acids are left. They are delimited by residues 15 to 19, 32 to 35, and 54 to 64, respectively. Mutagenesis studies of the B subunit have indicated that substitutions at positions 16, 17, 30, 33, and 60 either abolished or reduced the cytotoxic potential of the resulting toxin while an Asp to Asn substitution at position 18 altered the receptor specificity of the toxin (85-89). Molecular modelling studies involving the docking of CD77 (Gb_3) to the B-subunit have implicated residues located in these loops (90,91). It has been hypothesized that there are two potential binding sites for CD77 on the B subunit pentamer, namely, sites I and II (90,91). Residues located in regions 15-19 and 30-33, in particular Asn15, Asp 16, Asp 17, and Phe 30, form most of the putative binding site I (91). The calculated interaction energy derived from modelling studies suggested that site I is likely to be the predominant site mediating CD77 interaction (91). Thus, results from both site-directed mutagenesis and docking experiments suggest that residues found in loop regions are sites where random mutagenesis may lead to an altered receptor specificity. As described herein, residues are perturbed within two loop regions. namely, residues 15-19 (loop 1), and residues 30-33 (loop 2; technically speaking, this region is not a loop but rather represents the end of the ß4 strand and the beginning of the second loop). Random mutagenesis in loop 3 (residues 58-64; Fig. 2) may also be effective in achieving the objective of the invention. Though initial studies have focussed on the aforementioned regions of the molecule, this delimitation does not preclude the

possibility of targetting any of the B subunit residues in attempts to alter specificity of the toxin.

Nine residues are involved in loops 1 and 2, creating a potential library complexity of the order of 20^9 (5×10^{11} different mutant proteins, if all nine residues were totally randomized and all potential combinations recovered). It is, therefore, advantageous to reduce the level of complexity of the toxin library so that the nine residues of interest are not completely randomized. This goal was accomplished by synthesizing oligonucleodites for use in the mutagenesis procedure that have increasing levels of nucleotide "doping". The selection of an oligonucleotide with the desired level of doping for mutagenesis subsequently allows direct control over the level of diversity in the library made from that particular oligonucleotide pool. For example, mutations at 5 amino acid positions out of 9 in the target region, would yield a diversity of the order of 20^5 (3.2×10^6 mutant toxins), a more satisfactory level of diversity. Indeed, the screening of libraries with greater than 10^6 compounds has not previously proven necessary for chemical or peptide libraries in terms of identifying useful "lead" compounds (using either binding assays or functional assays in the screening process). Additionally, the number of potential target sites on cell surfaces will be large and will increase the need for screening steps.

Templates and Primers

Shiga and Shiga-like toxin 1 differ in sequence by only one amino acid in their A subunit and have identical B subunits. Although the random mutagenesis procedures described herein use the SLT-1 operon, the simpler terminology "Shiga toxin library" has been used rather than "Shiga-like toxin 1 library" in defining an ensemble

of mutant proteins derived from the Shiga toxin structural template.

Briefly, the recombinant plasmid pJLB28 (32) was used as a template for mutagenesis. This construct carries a *BglII-BalI* fragment of bacteriophage H-19B inserted in pUC19, which specifies for the production of active SLT-1 holotoxin. An additional construct was made by cloning a PCR product consisting of the SLT-1 operon carried by pJLB28 into the prokaryotic expression vector pTUG (92). The latter construct, pTGXH, encodes for the production of SLT-1 with a hexa-histidine sequence fused to the N-terminus of the A chain, to facilitate the purification of toxin variants. There are numerous methods available for generating random mutations in DNA. Mutagenesis using synthetic oligonucleotides with regions of defined degeneracy (93-96), is an established and reliable technique which satisfies the requirements of the invention, i. e., a rigidly defined mutagenic window and the need to control the frequency and type of mutations generated. Mutagenic oligonucleotides (98-mers) with the sequence indicated in Fig. 3 were synthesized on an Applied Biosystems 392 DNA synthesizer. The primers were designed to mutagenize both loops 1 and 2 simultaneously. A silent mutation introducing a new Sac I restriction site between the two zones was incorporated into the mutagenic primer to facilitate screening of transformant DNA and to allow for the "shuffling" of zones between variants. Five different (98-mers) mutagenic primers were synthesized with increasing levels of "randomness" in loops 1 and 2, so that libraries of predictable size could be generated. This strategy was accomplished by synthesizing codons in the loop regions in the form "NNS", where N is a base added to the growing chain from a mixture of the wild-type base "doped" with a fixed percentage of the three other bases, and S is a base added from a 1:1

mixture of cytosine and guanine. The latter aspect of the method allows codons specifying all 20 amino acids, but makes the chances of observing a given amino acid closer to 1:20 by reducing the degeneracy of the DNA code. Also, only the amber stop codon TAG can be generated using this strategy; thus, minimizing the production of truncated proteins.

The five mutagenic primers synthesized had doping levels ranging from 1.2% to 75%, where 75% represents completely random codons (*i. e.*, the phosphoramidite mixture used to place the given base contained 25% wild-type bases and 25% each of the other bases). A mutagenic primer made with a 12.5% doping level was chosen for initial studies to produce a library where the number of potentially different sequences (3.2×10^6 mutants, or a mutation rate of approximately 5 substitutions out of 9 per clone) was well within the limits of *Escherichia coli* transformation efficiency.

Mutagenesis

Two strategies have been employed so far to incorporate the mutagenic oligonucleotides into the toxin operon to create libraries of variant proteins; using the unique site elimination method (97) or by creating a combinatorial cassette. Single-stranded random mutagenic primers were incorporated into double-stranded plasmids using the unique site elimination (USE) mutagenesis method (97) employing the Pharmacia USE kit. This method allows mutagenesis to be performed on any double-stranded plasmid in the absence of restriction sites (97).

In an attempt to increase the efficiency of the mutagenesis procedure and to maximize the diversity of clones obtained, a combinatorial cassette method has also

been employed to generate toxin libraries. In this method, the same oligonucleotid pools depicted in Fig. 3A were annealed to an overlapping oligo sequence shown in Fig. 3B. A double-stranded cassette was created by mutually primed synthesis, i.e., by including DNA polymerase and dNTP's in a reaction with the overlapping pair such that each oligonucleotide would code for the formation of the opposite sense strand. The cassette was then amplified using PCR and cloned directly into the vector containing the toxin operon at sites AccI and PstI.

Further refinements to the mutagenic process are currently ongoing, and libraries are now being created using an entirely ligation-free system employing the uracil DNA glycosylase method (101). Notably, the demonstrated ability to use the same random oligonucleotide pools in a variety of different mutagenesis procedures underscores the flexibility of the system and its high capacity for adaptation and rapid improvement.

Screening

An initial library was constructed using the USE method with a mutagenic oligonucleotide with a 12.5% doping level. Following transformation of *E. coli* strain JM101 with vector DNA within which the randomized oligonucleotide had been incorporated, colonies on agar plates were grown in 96-well plates with conical well-bottoms and individual clones were picked from isolates. To confirm that the variants were producing toxin with an A chain capable of inactivating ribosomes, extracts produced by 17 clones selected at random were collected and assayed for their ability to inhibit eukaryotic protein synthesis. This assay uses Promega

TnT coupled transcription/translation reticulocyte lysate system, and consists of measuring the product of a luciferase gene in the presence and absence of bacterial extracts. The extracts of all the clones tested inhibited translation of the luciferin protein. Five of these variants were sequenced, and the nucleotide sequences of the randomized loop regions are listed in Table 1. Although the sample size is far too small at the present time to make accurate conclusions, the clones tested reflect the desired rate of mutation of approximately 5 out of 9 amino acid changes per clone.

Clone	Loop 1	Loop 2	Mutation Rate
Wild-type	AAT GAT GAC GAT ACC	TTT ACC AAC AGA	
ShT	N D D D T	F T N R	
#6	AAC GAG GAG GAG ACG	TTC GCG AAC AAC	
	N E E E T	F A N N	5/9
#13	AAC GAG CAG GAC ACC	TTC ACC CAC AGG	
	N E Q D T	F T H R	3/9
#15	AAG GAG AAC GAG AGC	TTC GCG AAC AAC	
	K E N E S	F A N N	7/9
#17	AAG GAC GAC GCG AGG	TTG ACC CAG AGG	
	K D D A R	L T Q R	5/9
#19	AAG GAC GAC GAC ACG	TTG ACC CAG AGG	
	K D D D T	L T Q R	3/9

Table 1. Comparison of nucleotide and amino acid sequences between mutagenic loops of five ShT mutant clones recovered from one of our ShT combinatorial libraries (12.5% doping level) and wild-type Shiga toxin. Loops 1 and 2 represent residues 15-19 and 30-33 of the B subunit of ShT (or SLT-1) respectively.

Cell cytotoxicity as a screening assay

The ability of a ShT variant to kill cells represents the most direct and practical measure of its utility. This function (cytotoxic property retained by all toxin variants) provides each mutant with a built-in search engine allowing one to screen any ShT combinatorial libraries against any eukaryotic cells to

identify novel mutant toxins that can kill such cells. The breast cancer cell line SKBR3 was used as the initial eukaryotic target since one of the long-term objectives of the invention is to develop anticancer agents for breast cancer. A set of 1000 clones were picked from the (12.5% doping) library to test the screening strategy. An 8 x 8 sib selection grid system (98) was used, whereby a given clone was pooled with seven others in a system where every clone tested was present in two separate pools. The 8-clone pools were amplified and then extracts from the mixtures were tested for cytotoxicity on Vero cells (a cell line highly susceptible to the wild-type toxin) and the human breast cancer SKBR3 cell line (a cell line than is insensitive to the wild-type toxin). A colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells was used to quantify cell viability. The cleavage of WST-1 gives rise to a water-soluble formazan that can be readily measured in the visible range (450 nm) using a 96-well plate format and a plate reader; thus, allowing the use of high throughput screening approaches. Other colorimetric cell viability assays were or could be used such as alternate tetrazolium salts XXT, MTT, or dyes such as sulforhodamine B. In addition, screening could be performed using cell proliferation assays measured in terms of counting cell colonies or the incorporation of radiolabeled nucleotides or amino acids into nucleic acids or proteins. Clones that were implicated in producing cell-killing toxins were retested individually on the same cell lines. This preliminary set of clones has yielded thus far at least 14 clones that show a dramatic increase in their ability to kill SKBR3 cells relative to the wild-type ShT (Table 2), and several clones that show reduced cytotoxicity on Vero cells but enhanced SKBR3 toxicity. The latter clones are of significant interest, since the goal of the invention is

to alter the natural specificity of the toxin from the CD77 glycolipid to another cell surface marker. On the basis of data so far obtained, scaling up the screen to greater than 1000 single clones is being done in order to optimize the screening strategy. The immediate aim is to reach a minimal screening level of 10^4 single clones.

CLONE	LOOP 1					LOOP 2			
ShT wild-type	N	D	D	D	T	F	T	N	R
SKBR-3									
66	N	E	E	E	T	E	F	T	G
110	N	D	D	D	T	F	T	K	S
128	T	T	D	D	P	G	T	R	G
220	N	D	D	D	T	L	T	N	G
241	N	D	D	D	T	F	T	K	S
256	N	D	D	D	T	L	P	N	R
265	N	D	D	D	T	F	T	N	C
415	K	E	D	E	S	L	T	K	R
506	N	D	D	D	T	L	T	K	S
619	Y	D	D	N	P	L	T	N	S
766	N	D	D	D	T	L	T	K	R
767	K	K	E	E	P	C	A	N	R
A22	N	D	D	D	T	L	T	K	R
A25	N	D	D	D	T	L	T	N	R
CAMA-1									
*308C	P	Y	V	F	L	M	V	A	N
*241C	F	R	P	A	G	L	R	C	G
*142C	T	G	A	T	M	P	T	G	I

Table 2. Amino acid sequences of clones exhibiting cytotoxic activity on SKBR-3 cells (recovered from a 12.5% doping level library) and CAMA-1 (clones recovered from a 75% doping level library). Loops 1 and 2 represent the same B subunit residues indicated in Table 1.

A second library, this time using an oligonucleotide pool with a doping level of 75% (i.e., completely randomized) was created using the combinatorial cassette method described previously. The library was screened essentially as the first using the sulforhodamine B cell viability assay (a less expensive and more reliable assay than the previous WST-1 assay), and the cell line CAMA-I. This cell line is also a breast carcinoma like SKBR-3, but has been shown to lack the CD77 marker and is extremely resistant to the native SLT-1 toxin. One thousand individual clones from the cassette library were screened for cytotoxic effect on CAMA-I, and as in the case of SKBR-3 several promising toxin variants were indentified, whose sequences are shown in Table 2. As expected, the clones identified from this highly diverse library harbour an almost completely random array of amino acid substitutions in their mutagenic regions.

In light of the demonstrated utility of the invention, the skilled person will appreciate that the method can be applied to other cell lines with the expectation that useful therapeutic and diagnostic molecules will be identified. With numerous target sites on cells, it is expected that a large number of mutant toxins will be found with cytotoxic activity.

To reduce the requirement for a uniquely selective agent for cancer cells, a major concern in the design of *in vivo* treatment strategies, it may be advantageous to evaluate the utility of toxin variants in the context of *ex vivo* purging situations. Their value can be readily assessed by exposing bone marrow cells or peripheral stem cells to these agents and observing the level of reconstitution of haematopoietic cell lineages using flow cytometry under *in vitro* or *in vivo* settings (transplantation experiments in SCID, NOD/SCID mice, for

example; ref. 14). The initial selection of breast cancer cell lines SKBR3 and CAMA-I as the target of the ShT library searches stems from the fact that most autologous bone marrow transplants (ABMTs) or peripheral stem cell transplantations are presently performed on breast cancer patients, and that an *ex vivo* purging of their stem cells may prove beneficial in terms of the patient's long-term survival.

Potential benefits and receptor diversity of Shiga toxin libraries

The construction of Shiga toxin libraries will permit one to rapidly identify new cytotoxic/diagnostic probes with altered receptor targeting properties. Since the natural receptor for the B subunit of Shiga toxin is a glycolipid, the specificity of mutant B subunits derived from libraries harbouring a low level of degeneracy in the sequence of its loops, may be directed at unique carbohydrate structures located on glycoproteins or glycolipids. In the case of toxin libraries containing highly degenerate sequences within the two loop regions, it is expect that the potential surface structures recognized will be very diverse. As in the case of antibody combining sites, B subunit variants may bind to a spectrum of molecular entities such as proteins, peptides, nucleic acids or even organic moieties rather than to sugars or glycolipids. The construction of Shiga toxin libraries offers several distinct advantages. **Firstly**, the libraries are permanent and can be indefinitely screened to provide a continual source of new therapeutic or diagnostic agents. **Secondly**, the lethal character of the resulting toxin mutants towards eukaryotic cells allows one to easily screen for useful constructs having a specificity for unique cell targets (such as cancer cells). **Thirdly**,

useful mutant B subunits can be generated in the absence of a cytotoxic A chain, permitting the immediate creation of non-cytotoxic diagnostic agents that can be used to detect the presence of unique markers on cell types in either *in vitro* or *in vivo* settings.

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A.

1
KEFTLDFSTAKTYVDSLNVIRSAIGTPLQTISSGGTSLLMIDSGSGDNLFAV

DVRGIDPEEGRFNNLRLIVERNNLYVTGFVNRTNNVFYRFADFSHVTFPGT

TAVTLSGDSSYTTLQRVAGISRTGMQINRHSLTTSYLDLMSHSGTSLTQS

VARAMLRFVTVTAEALRFRQIQRGFRTTLDDLSGRSYVMTAEDVDLTLN

WGRLSSVLPDYHGQDSVRVGRISFGSINAILGSVALILNCHHHASRVARM

293
ASDEFPSMCPADGRVRGITHNKILWDSSTLGAILMRRTISS

B.

1 15 19 30 33
TPDCVTGKVEYTKY[NDDDT]FTVKVGDKEL[FTNR]WNLQSLLLSAQITGMTV

58 64 69
TIKTNAC[HNGGGFS]EVIFR

FIG. 1

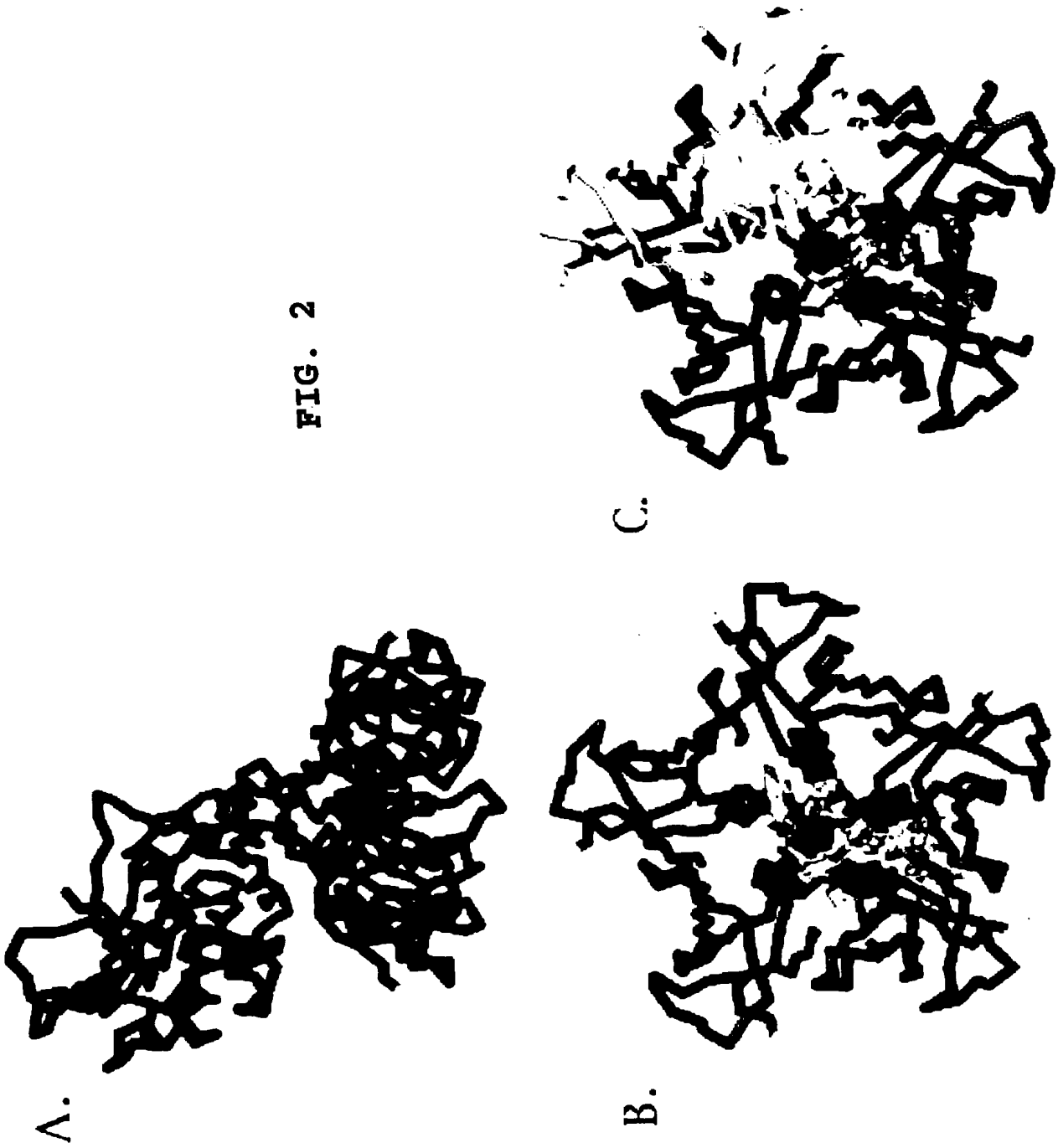

FIG. 2

A

Acc I **Loop 1**

5Õ-AAG GTG GA**G** **TAT** **AC**A AAA TAT AAT [NNS NNS NNS NNS NNS] ACA GTT AAA

Sac I **Loop 2**

GTG GGT GAT AAA **GAA** **TTA** [NNS NNS NNS NNS] TGG AAT CTT CAG TCT C

B

Pst I

5Õ-TAC GTA **CTG** **CAG** CTC GAG TCA ACG AAA AAT AAC TTC GCT GAA TCC ACC GCC ATT ATG GCA CGC GTT AGT TTT AAT GGT TAC AGT CAT ACC GGT AAT TTG CGC ACT GAG AAG AAG AGA CTG AAG ATT CC-3Õ

FIG. 3